ADAMA SCIENCE AND TECNOLOGY UNIVERSETY
SCHOOL OF APPLIED NATURAL SCIENCE &APPLIED CHEMISTRY PROGRAME

PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITIES ONTHE LEAVES EXTRACT OF CROTON MACROSTACHYUS (BIsana)

BY

MESFIN TESFAYE BELIHU

SEPTEMBER 2009

ADAMA SCIENSE AND TECNOLOGY UNIVERSETY, ADAMA

PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITIESON *THE LEAVESEXTRACT OF CROTON MACROSTACHYUS (BISANA)*

A Thesis Submitted to the Program of Chemistry,

Postgraduate Program

ADAMA SCIENSE AND TECNOLOGY UNIVERSETY

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE IN CHEMISTRY (ORGANIC CHEMISTRY)

Mesfin Tesfaye Belihu

SEPTEMBER 2009

ADAMA SCIENCE AND TECNOLOGY UNIVERSETY

SCHOOL OF APPLIED NATURAL SCIENCE &APPLIED CHEMISTRY PROGRAME

PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITIESON THE LEAVES EXTRACT OF *CROTON MACROSTACHYUS (BISANA)*

BY

MESFIN TESFAYA BELIUH
PROGRAME OF APPLIED CHEMISTRY
SCHOOL OF APPLIED NATURAL SCIENCE

SEPTEMBER 2009

APPROVED BY THE EXAMINING BOARD:

Dr. Amane Dekebo _____
Advisor
Dr. Haylemikael Tesso _____
Examine
Dr.Yadesa Melaku _____
Examiner
Dr. _____
Examiner
Dr. _____
Examiner

II

Dedication

To my wife and child for their co-operation at the time I was away adama science and Technology University in my M.S. study

Acknowledgements

First of all, I would like to thank the almighty God and his mother Merry who allowed me health and strength throughout my work.

I would like to express my deepest respect and most sincere gratitude to my advisor, Dr Aman Dekebo for his guidance, professional advice, constructive comments and encouragement of my work from the beginning up to the completion of this thesis. Also, I would like to thanks my examiners, Dr. Haylemikhael Tesso and Dr. Yadesa Melaku for their valuable guidance; constructive criticism and encouragement during every stage of this project

My heartfelt thanks go to Mr.Solomon Gidey head of Chemistry Department of Adama sience and Technology University. For allowing me to use laboratory and providing materials in my laboratory work. And my thanks go to his Secretary, for her respection and contribution.

I am forever grateful to my friends Dereja Tadese and Ambisa Melaku for their well understanding and support throughout the study period. Without their support, this work would not have been possible.

I would like to thank Oromia public Health Research, Capacity building & quality Assurance Laboratory Center, for enabling me perform bioassay tests. It is also our pleasure to thank for chemistry department laboratory technician for their supporting during our study time for laboratory analysis.

I want to express my deep gratitude to my families, specially my sistor, who has given me a constant source of love, sharing my trouble and invaluable supports in my M.S. study *and* I owe a debt of gratitude to Ephrem & Emuti in Boston(USA), for their support and encouragement both mentally and materially during very difficulty moments of this M.S. study, In addition, I would express my deep appreciation to my loving, supportive, encouraging and patient wife who accompanied me during my every stage of M. S. study

Lastly, but never the least, I would like to thank my son Natane, for his immeasurable sacrifices, endurance and understanding during my endless circles of hope and despair.

Table of Content

Table of contents (continued)

I am particularly grateful to Dr. Wondimagegn Mammo for his immediate response when support is needed and also for his consistent help and encouragement throughout the project work.

List of Tables

LIST OF FIGURES

LIST OF FIGURES IN THE APPENDIX

LIST OF ANNEXS

ACRONYMS AND ABBREVIATIONS

^{13}C NMR	Carbon -13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
CC	Column Chromatography
DEPT	Distorsionaless Enhancement by Polarization Transfer
FT-IR	Fourier Transform Infra-Red Spectroscopy
NMR	Nuclear Magnetic Resonance
Rf	Retardation Factor
TLC	Thin Layer Chromatography
UV	Ultra Violet
C	Croton
C. spp	Croton species
MDR	Multidrug resistant tuberculosis
MIC	Minimum Inhibitory Concentration
WHO	World Helth Organizaton
AAU	Addis Abeba university
MHA	Muler Hinton Agar

ABSTRACT

PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITIES ON THE LEAVES EXTRACT OF CROTON MACROSTACHYUS

By; Mesfin Tesfaye
Advisor: Dr. Aman Dekebo

Croton macrostachyus Hocsht. ex Del. (Euphorbiaceae) leaves is one of the 1200 species of Croton have been described with biological activities, mainly due to diterpenes, alkaloids and/or other secondary metabolites. These activities account for the traditional use of Croton species to treat certain diseases in South America, Asia and Western Africa. Hence the aim of the present study was to investigate phytochemicals of the plant leaves and evaluate the antimicrobial activity of its extracts against bacteria and fungi. The leaf powder Croton macrostachyus was successively extracted with n-Hexane, chloroform, ethyle acetate and methanol. The crude chloroform, ethyle acetate and methanol extracts obtained from leaves of Croton macrostachyus were subjected to phytochemical tests for the presence of secondary metabolites, tannins, cardiac glycoside, alkaloids and flavonoids.etc. In all extracts studied, Flavonoids, tannin, terpenoid, saponins, steroids, cardiac glycoside and alkaloids, were present in the, methanol and chloroform extracts while terpenoid and steroid were detected in chloroform crude extracts and essential oil. The antimicrobial activity of extracts was studied using the disk diffusion method and determination of minimal inhibitory concentration (MIC) value. The antimicrobial activity of C.macrostachyus leaves extracts and essential oil was tested against pathogenic bacteria like gram positive bacteria Staphylococcus aureus, Salmonella typhimurium, and gram negative bacteria Klebsiella pneumoniae, Escherichia coli and fungi like Candida albican. The highest antibacterial inhibition against S.aureus (14mm) and E. coli (12mm) were revealed by chloroform and methanol extracts reaspectively. While the least inhibition zone (8mm) has shown against S.aures by ethyle acetates. The antimicrobial assay showed that volatile oil was generally more active on E. coli (9 mm) than S. aureus (6 mm); whereas higher antifungal activity was observed on C.albicans (inhibition diameter (24.20 mm). Repeated use of solvent extraction followed by CC, leaf extract of C.macrostachyus yielded two compounds and only one characterized as *tetrahydro-2-hydroxymethyl) -6- (tetrahydro-3, 4,5-trihydroxy -6(hydroxymethyl)-2H-pyran-2-yloxy) -2H-pyran-3,4,5 -triol, whose structure was elucidated based on its ^{1}H-NMR, ^{13}C-NMR,FT-IR and DEPT data. Crude extracts and essential oil exhibited reasonable antimicrobial activities. These showed potential as antimicrobial agents and might be used to improve therapeutic efficiency to overcome, rising bacterial and fungal resistance of existing drugs.This study provides some scientific base for the folkloric medicinal use of this plant as remedy for ailments whose causative agents are the pathogens studied. The activities observed could also be attributed to the phytochemicals detected, some of which have been associated with antimicrobial activity.*

Keywords: *antimicrobial activity, phytochemical investigation C. macrostachyus (Euphorbiaceae), Gram-positive organism, Gram-negative organism,*

Chapter 1 – Introduction

1.1 Medicinal plants

Historically, some plants have played a significant role for traditional medication. Through observation and experimentation, human beings have learnt about some traditional medicinal plants which used to promote health. The use of these traditional medicinal plants as remedies is not only cost effective but also safe for health. They are also almost free from serious side effects. These traditional medicines are used as health practices, approaches, knowledge and beliefs. They are obtained from plant, animal and mineral based which used for spiritual therapies, being the plants are the most important source for medication. They used to apply singularly or in combination to treat, diagnose and prevent illnesses and maintain well-being (WHO 2001, Mazid, *et.al*. 2012).

Since the people did not have the scientific insight to explain and predict the curative action of plants, in some countries, the use of traditional medicinal plants is often associated with witchcraft and superstition. One example of such an irrational concept is the Doctrine of Signatures, elements of which are found in many of the healing cultures of the world. It is based on the assumption that the appearance of plants may give clues to their medicinal properties. It is interpreted as God's signature on the plant. For instance, Red juice and sap, is associated with blood and menstrual ailments. The yellow flower and alkaloid containing latex of some plant associated with bile and jaundice. It is crude extract used successfully to treat jaundice. The human shape of certain roots also associated with the female form of fertility (Gurib-Fakim, 2006).

Several species of Euphorbiaceae are known in many parts of the world for their toxic and/or medicinal properties (Salatino *et al.,* 2007). Such diversity of effects is a reflection of the variety of secondary metabolites which have already been described for this family.Croton is one of the giant genera of angiosperms, comprising over 1200 species of herbs, shrubs, trees and occasionally vines. Recent studies have estimated that approximately two-thirds of the *Croton* species occur in the New World, the other third being distributed across the Old World (van Ee *et al*., 2011). It is the second largest genus in Euphorbiaceae, with various species used in folk medicine in many regions of the world.

The predominant genera under the Euphorbiaceae family are Drypetes, Jatropha, Macaranga, *Croton*, Euphorbia, Acalypha, Glochidion and Macaranga.A number of the Croton species are known for their medicinal qualities especially in Africa, Asia and South America. *Croton* has been found to possess secondary metabolites such as alkaloids, terpenoids, flavanoids and compounds such as diterpenoids. Croton spp. are commonly used for the treatment of non communicable diseases such as diabetes, cancers and other ailments such as digestive problems, dysentery, wounds, fevers, constipation, diarrhea, intestinal worms, malaria, pain ulcers, inflammation. The parts that areused for the treatments of the different kinds of disease are the leaves, the roots, the stem barks, the fruit and the seeds (Yibralign 2007).

Based on the medicinal properties of plants of Croton genus, the present study aimed to investigate the antimicrobial activity of the extracts isolated from *Croton macrostachyus* belongs to the family Euphorbiaceae. The plant was selected for this study, because it is used as traditional medicine in Negelle-Borena Gujii Zone. The people in these area recommend it, especially its leaf juice, ‘‘for healing wounds and blood clotting’’. Although, the people use this plant as a traditional medicine for blood clotting and healing wounds, still the chemical component of the plant was not studied. As a result, the researchers are intended to perform chemical and antimicrobial study on the leaves of plant.

1.2 Traditional medicine

Traditional medicine is a vague term, loosely used to distinguish culture-bound health practices from modern or allopathic medicine *(WHO, 2003).* However, traditional medicine can simply be defined as the application of indigenous beliefs, knowledge, skills and cultural practices concerned with human health *(Balunas and Kinghorn, 2005)*. Some frequently used synonyms are indigenous, unorthodox, alternative, folk, ethno and unofficial medicine. And the use of the term "modern" in reference to non-traditional medicine was advanced on the basis of its tendency to quickly adopt scientific and technological innovations, an advantage that is employed by traditional medicine to a very small extent both traditional and modern medicines have a common objective of healing and preventing disease. However, the two systems differ in the concept of the cause of disease, their approach to healing as well the methods of treatment. Although traditional medicine mostly employs the use of plants or plant parts *(ABEBEand*

AYEHU 1993), may be used. Traditional medicine is wide spread throughout the world and in fact, it was the only form of health care before the incorporation of modern science into medicine in the early part of the 20th century. Its part of the traditions of each country and its practices are handed downfrom generation to generation. Moreover, there is a revitalization of interest in herbal products at a global level and the conventional medicine is now beginning to accept the use of scientifically validated botanical source of drugs (*Gilani&Rahman, 2005)*..

1.3 Justification of this study

The *Croton* species are found in different parts of the world and are known for their medicinal properties. Popular uses of Croton include treatment of malaria, fever, external wounds, dysentery, and digestive problems. (Salatino *et al*., 2007). Some species of *Croton* are known to contain *alkaloids*. Diterpenes a common compound in *Croton* may be represented *as* clerodanes, cembranoid, halimanes, kauranes, labdanes, phorbol esters, trachylobanes and sarcapetalanes. Volatile oils are present in some of the Croton species (Salatino et al. 2007). *Some of these compounds present* in Croton *species have been shown to have antimicrobial properties (Peres* et al. *1997, Abo* et al. 1999). *Evidence from in vitro studies point to the use of essential oils as antimicrobial agents for wide range of pathogenic bacteria strains such as E. coli, Shigella dysenteria, Bacillus cereus, S.aureus and Salmonella typhimurium (Burt* 2004, *Nguefact et al*. 2004, S*chmid*t et al. 2005). Therefore. The presence of these important phytochemicals in the plant is a scientific justification of the plant use in the traditional treatment against various diseases affecting humans and animals.

1.4 Statement of the problem

Infectious diseases which include typhoid, pneumonia and candidiasis. The Centre for Disease Control and Prevention (CDC) has proposed monitoring and development of new treatments which encompasses the development of new antimicrobials (Fauci, 1998). Is the leading cause of death worldwide, accounting for nearly one half of all deaths in tropical countries which are also becoming a significant problem in all countries? Microbial infections are great challenge to human health concern and it is even exacerbated by the growing resistance to the conventional drugs. Thus, researchers have resort to find remedy from plants for infectious diseases. Development of new antimicrobial agents is among the proposed solution to solve this problem. In this regard plants can be provided a good alternative in search for new chemicals with a wide range of antibacterial and antifungal activities (Casley-Smith, 1997). This study aimed at screening the leaves part of *C.macrostachyus* for antimicrobial activities, in order to fill the gap in knowledge of their efficacy.

1.5 Objective of the Study

1.5.1. General objective

To investigate the phytochemicals and antimicrobial activities of extract of the leaves of *Croton macrostachyus.*

1.5.2. Specific objectives

- To extract and isolate phytochemicals from leaves of *Croton macrostachyus* using different techniques
- To extract essential oil from leaf of plant using hydro-distillation .
- To test the antimicrobial activities of crude extracts, (chloroform, ethyle acetate and methanol) and essential oil from the plant by disc diffusion method.
- To characterize the isolated compounds using different spectroscopic techniques ^{1}H NMR, ^{13}C-NMR, FT-IR and DEPT-135).

Chapter 2 - Literature Review

2.1 Drug resistance pathogens in this study

Different bacteria and fungai were used in the referred studies they were *Staphylococcus aureus, Estrichiacoli, Salmonella typhimurium, Salmonella typhosa, Klebsiella pneumoniae,*For infections to occur, bacterial adhesion is required as well as the host cell surface carbohydrates, which serve as receptors for adhesion. Adherence of bacterial cells to specific receptors such as glycoconjugates and extracellular matrix molecules prevents dislodgement by the host mucociliary defense mechanisms and aid bacterial invasion into cells (Kouki*et al*. 2011). *E. coli* adhesins bind to the *galactosyl-1–4-galactose* in the host receptor cells. Thus *E. coli* causes many infectious diseases in human such as urinary tract infections and common intestinal diseases (Matias*et al*. 2011).

The genus *Staphylococcus* is distributed in the environment and is often etiological agent for opportunistic infections in humans and animals. *S. aureus, Listeria monocytogenes, Listeria innocua, Salmonella typhimurium, E. coli, Shigella dysenteria, Bacillus cereus and Staphylococcus aureus (*Burt 2004*).* Biologically active compounds such as *sonderianin, korberin* A and B isolated from the *genus Croton* with specific antibacterial activities *against Mycobacterium smegmatis, Staphylococcus aureus, Bacillus subtilis, E. coli (*McChesney *et al.* 1991).

Among the harmful microorganisms to man are the pathogenic *fungi, bacteria*, which are causative agents for various diseases. Man has therefore developed conventional medicine through which these microbes and the diseases they cause are controlled. However it is evident that modern medicine does not provide the cure to some of the known human diseases. Even though a number of new antibiotics have been produced by pharmacological industries in the last three decades, resistance to these drugs by micro-organisms has increased tremendously. Bacteria for instance, have the genetic ability to transmit and acquire resistance to drugs which are utilised as therapeutic agents (Cohen, 1992).

This resistance is mediated by (i) the acquired genes, whose presence in a cell is usually synonymous with a resistance phenotype; (ii) mutations in resident genes that alter a variety of phenotypes, including alteration of target sites and enhanced efflux mechanisms; or (iii) changes in outer membrane proteins, which limit the access of drugs to the cell (Rasheed and Tenover, 2003).

The resistance factor in these micro-organisms is a cause for concern, considering the number of patients with suppressed immunity, and the emergence of new multi-resistant bacterial strains.In

Kenya, *(*Okemo *et al.,*(2004*)* established that the *S. typhi* causing typhoid is a multi-drug resistant (MDR) strain. Similarly, *Shigella*serotypes isolated from patients with diarrhoea in Kwale district showed that 29% of *S. Flexneri were* resistant to tetracycline. Multi-drug resistant enterotoxigenic, enteroinvasive and enteropathogenic E. coli were isolated from 346 children with diarrhoeagenic *E. coli*, in Tanzania (Vila, 1999*).* These strains were resistant to ampicillin, clotrimoxazole and chloramphenical. These current trends call for renewed strategies in microbial therapy. This involves the development of new treatments, which encompasses development of new antimicrobial agent (Abo *et al.* 1999)

2.2. Phytochemicals present in medicinal Plants

Medicinal plants are rich source of wide variety of secondary metabolites belonging to chemical classes such as *sterols, alkaloids, glycosides, saponins, flavonoids, tannins, and carbohydrates* are generally superior in their anti-microbial activities (Cowan, 1999).These cases of compounds have been found to inhibit among other micro-organisms, *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. Other antimicrobial compounds isolated from plants include coumarins, steroids, crinitol, papaverine, anonaine and mannitol which have inhibitory effects *on B. subtilis, S.aureus, C. albicans, C. utilitis, measles*, and HIV (Taniguchi and Kubo, 1993; Kubo *et al*., 1992).

Different phytochemicals display various mechanisms of action such as increasing colonic water and electrolyte re absorption and inhibiting intestinal motility, while some components have been shown to inhibit specific pathogens (Ahmad *et al*., 2006). *Saponins* are used by the folkloric remedies of Kashmir (India) in treating wounds, help in blood clotting and enteric ulcers problems (Foster and Duke, 1990). This is because of their ability to cause red blood cells to precipitate and coagulate (Just *et al.,* 1998). The presence of *saponins* shows the potential of the plants to be used to produce mild detergents and intracellular histochemistry staining to allow antibody access to intercellu-lar proteins (Just *et al.,* 1998).

Tannins have stringent properties, hasten the healing of wounds and inflamed mucous membrane (Njoku and Akumefula, 2007). Their astringent property makes them useful in prevent-ing diarrhea and controlling hemorrhage due to their ability to precipitate proteins, mucus and constrict blood vessels. This is the reason why traditional healers use plants reach in tannins to treat wounds and burns since they are able to cause blood clotting. Some *tannin*has being

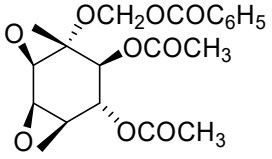
reported to inhibit HIV replication selectively besides the use of diuretics (Argal and Pathak, 2006). This shows how traditional medicinal plants rich in tannins can be used to control this dangerous disease. According to (Chung *et al.*,1998). Many tannin mole-cules have been shown to reduce the mutagenic activity of a number of mutagens. The growths of many fungi, yeast, bacteria and viruses have been proven to be inhibited by tan-nins.

Flavonoids **a**re important group of polyphenols widely distributed among the plant flora. Stucturally, they are made of more than one benzene ring in its structure (a range of C15aromatic compounds) and numerous reports support their use as antioxidants or freeradical scavengers (Kar, 2007). The compounds are derived from parent compounds knownas flavans. Over four thousand flavonoids are known to exist and some of them arepigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoidspresent in nearly 70% of plants. Other group of flavonoids includes flavones, dihydroflavons, flavans, flavonols, and anthocyanidins.

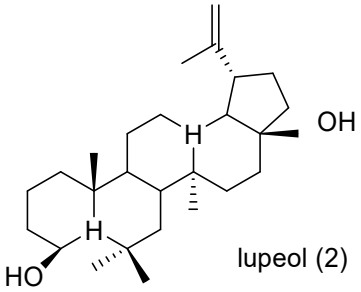
Flavonoids are known to contain specific compounds called antioxidants which protect hu-man, animal and plant cells against the damaging effects of free radicals. Imbalance between free radicals and antioxidants leads to oxidative stress which has being associated with in-flammation, autoimmune diseases, cancer, Parkinson's disease, aging and arterioscle-rosis. It also plays a role in heart diseases and neurodegenerative diseases. *Flavonoids* have also vasodilator activity a property which is useful in improving blood circulation in brain and in Alzheimer disease *(*WHO 2001, Mazid*, et.al.* 2012).

Terpenoids; have medicinal value such as *(anticarcinogenic,antimalarial,antimicrobial and diu-retics activity). Terpenoids* have also shown a great potential in treatment against disease causing microorganisms.*Terpenoids* have exhibited antibacterial activity against *E. coli, Staphylococcus, Pseudomonas aeruginosa (Kouki et al. 2011). Methillin-resistant S. aureus, Proteus mirabilis (Santos et al., 2008), Klebsiella pneumoniae (Santos et al.,*2008*). Listeria monocytogene, Enterobacter cloacae, yeast Candida albicans, Aspergillusflavus (Leandro and Vargas, 2012). Some Terpenes isolated from Croton macrostachyus including, Crotomacrine, Crotepoxide, 3β-acetoxy tetraxer-14-en-28-oicAcid, Trachyloban-18-oic acid, Lupeol.* trachylina-18-oic acid(**8**),

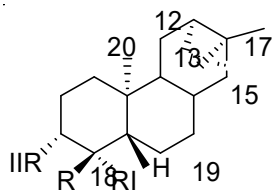
neoclerodan-5,10-en-19,6β; 20,12-diolie (**9)** 3α,19-dihydroxy trachylina (**10**), 3α,18,19-triydroxy trachylina (**11**). Menberu D. M.Sc (1981)



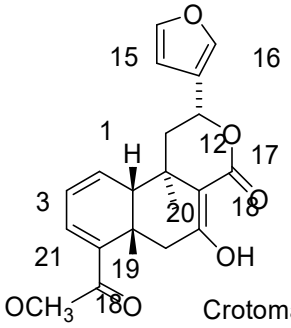
crotepoxide (1)[



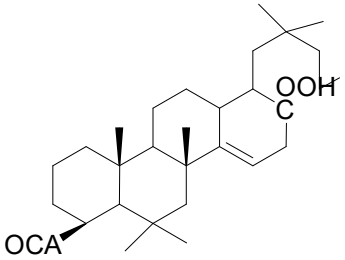
lupeol (2)



trachylina-18-oic acid



Crotomacrine(6)



3 β -acetoxy tetraxer-14-en-28- oic acid

Alkaloidsare the largest group of secondary chemical constituents made largely of ammonia compounds comprising basically of nitrogen bases synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptidering, most containing oxygen. The compounds have basic properties and are alkaline inreaction, turning red litmus paper blue. In fact, one or more nitrogen atoms that are present in an *alkaloid,* typically as 1°, 2° or 3° amines, contribute tothe basicity of the *alkaloid*. The solutions of alkaloids are intensely bitter. These nitrogenous compounds function in the defence of plants against herbivores and pathogens, and are widely exploited as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities. More than 12,000-*alkaloids* are known to exist in about 20% of plant species and only few havebeen exploited for medicinal purposes).

Quinones are another class of compounds, which are characteristically highly reactive. They are responsible for the brown-ing reaction of cut or injured fruits and vegetables and are known to complex irreversibly with nucleophilic amino acids in proteins often leading to inaction (*Stern et al.,* 1996). For that rea-son, the potential range of *Quinone* anti-bacterial effects is great.

Anthraquinones; these are derivatives of *phenolic* and *glycosidic* compounds. They are solely derived from anthracene giving variable oxidized derivatives such as *anthrones and anthranols (*Maury *et al.,* 2008; Firn, 2010). Other derivatives such as *chrysophanol, aloe-emodin, rhein, salinosporamide, luteolin* and *emodin* have in common a double hydroxylation at positions C-1 and C-8.Basic structures of somepharmacologically important plant derived anthraquinones found in *C. macrostachyus* plant.

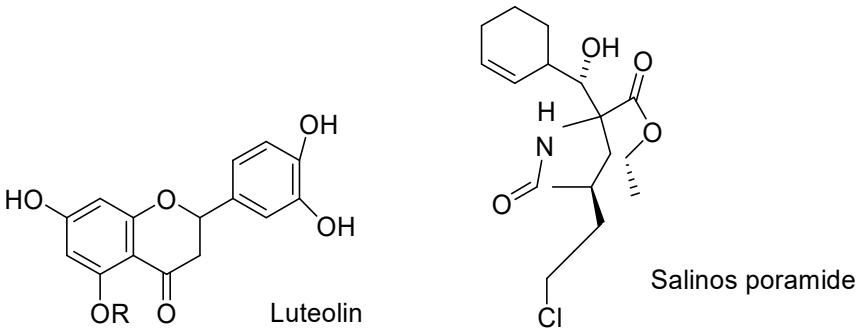
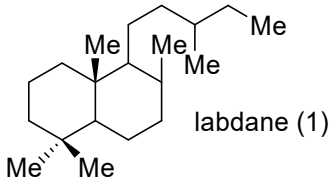


Fig-2 Some compounds from ***C. macrostachyus*** plant

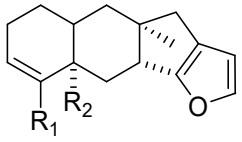
According to the WHO, medicinal plants would provide the best source to obtain a variety of drugs (Santos *et al.,* 1995*).* About 80% of individuals in developing countries use traditional medicine which has compounds derived from medicinal plants. Such compounds as *tannins, terpenoids, alkaloids, and flavonoids,* which are produced by plants as secondary metabolites have been found in vitro to have antimicrobial properties (Cowan, 1999). Since many of these compounds are currently available as unregulated botanical preparations and their use by the public is increasing rapidly, clinicians need to consider the consequences of patients self-medicating with these preparations. Therefore there is need to investigate such plants to understand better, their properties, safety and effica*cy.*

2.3 The genus Croton

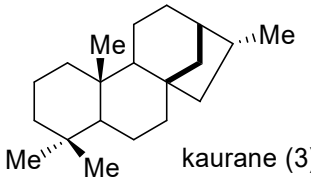
Croton is an extensive flowering plant genus in the' spurge' family, Euphorbiaceae. The common names for this genus are **rushfoil** and *croton*, but the latter also refers to *Codiaeum variegatum*. The generic name comes from the Greek κροτον (*kroton*), which means "tick" and refers to the shape of the seeds of certain species. *(*Gledhill, D. (2008) The genus *croton* is particularly rich in secondary metabolites like alkaloids, Terpenoids, and flavonoids. The most common class of compounds of *croton* is represented by *diterpenoids.* Apparently, *clerodane* is the widest spread class of *diterpenoids* in *Croton,* which has been found in species from America (e. g. C. cajucara), Africa (e. g. C. macrostachyus) and Asia (e. g. C. tiglium). The genus is also rich in Constituents with biological activities, chiefly *diterpenoids* such as *labdane* (**1**) *clerodane* (**2**), *kaurane* (**3**), *trachylobane* (**4**) and *pimarane (**5**).*



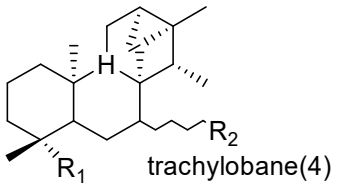
labdane (1)



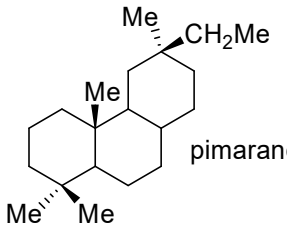
clerodane(2



kaurane (3),



trachylobane(4)



pimarane (5).

As most *Euphorbiaceae, Croton* species may contain *latex,* which is a characteristics of medicinal properties. Several *Croton* species have a long role in the traditional use of medicinal plants in Africa, Asia and South America. Popular uses include treatment of cancer, constipation,

2.4 Medicinal Importance of *Croton* Species.

Croton can be a tree, shrub or herbaceous plant which grows in tropical and warm regions. The genus *Croton* belongs to the subfamily *Crotonoidea*e of family Euphorbiaceae, one of the largest families of plants, often characterized by being monoecious. The predominant genera under the Euphorbiaceae family are *Drypetes, Jatropha, Macaranga, Croton, Euphorbia, Acalypha, Glochidion* and *Macaranga*.

A number of the *Croton* species are known for their medicinal qualities especially in Africa, Asia and South America. *Croton* has been found to possess secondary metabolites such as *alkaloids, terpenoids, flavanoids* and compounds such as *diterpenoids. Croton spp.* are commonly used for the treatment of non communicable diseases such as diabetes, cancers and other ailments such as digestive problems, dysentery, wounds, fevers, constipation, diarrhea, intestinal worms, malaria, pain ulcers, inflammation. The parts that are used for the treatments of the different kinds of disease are the leaves, the roots, the stem barks, the fruit and the seeds (Yibralign 2007).

Some of the *Croton spp*. are found in different regions*; C. zambesicus(Tannins Saponins,Anthraquinones ,Alkaloids) (*Abo *et al.* 1999*)* in Nigeria*; C. macrostachyus (alkaloids, terpenoids , Saponins ,flavanoids and tanine)(*Wagate *et al.* 2010*),* in Kenya*; C. tiglium*

proteins)(Shalid et al. 2008) Pakistan; C. campestris (Tannin, phlobaphenes, Flavones, Flavonols, Xanthones,Chalcones,Aurones,Flavononols,Catechins,Flavonones, Alkaloids and *Terpenes)* (Matias *et al.* 2011)*, C. zehntneri (*Rodrigues *et al.* 2009*),* C. cajucara (Alviano *et al.* 2005*),C. urucurana (Acetyl aleuritic acid β-sitosterol-O-glucoside Sonderianin Steroids (stigmasterol, β-sitosterol, campesterol) CatechinGallacatechin)(*Peres *et al.* 1997*);* and *C. sonderianus (ent-Beyer-15-en-18-oic acid*)(McChesney *et al.* 1991) in Brazil

2.5 Botanical description of *Croton macrostachyus.*

Selection of candidate plant species for biological activity screening can be approached in various ways. They include: a chemotaxonomic approach which is based on correlation between plant taxonomy and the occurrence of specific chemical compounds, random selection approach in which plants or plant parts are randomly selected and then subjected to biological screening and ethnbotanical approach in which plants used in traditional medicine are screened for biological activity (Liu 2011). Several researchers have reported that the ethno botanical approach is the best option.(Chariandy et al. 1999). The scientific discipline, ethno botany, is utilizing the impressive array of knowledge assembled by indigenous peoples about the plant and animal products they have used to main-tain health.

Plants also known to have special ability to synthesize aromatic substances, most of which include alkaloids, quinones, flavones, tannins, phenols or their oxygen-substituted derivatives *(Agrawal et al., 2012).*. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active. *(Liu, 2004).*

The genus Croton belongs to the family Euphorbiaceae (which commonly known as the 'spurge' family) (Negash, 2010). It is known as Bisana' (in Amharic) (Karunamoorthi and Ilango, 2010). and consists of approximately 1300 species of trees, shrubs and herbs distributed in tropical and subtropical regions of the world (Salatino *et al*., 2007).

Croton macrostachyus which is called 'rush foil' or 'broad-leaved croton is a multipurpose, medium sized, drought-deciduous pioneer tree. It is a tall tree found in tropical regions of

Africa. Elsewhere in Africa, *C. macrostachyus* has been reported to occur in Angola, Burundi, Cameroon, Central Africa, Ghana, Guinea, Ivory Coast, Kenya, Malawi, Mozambique, Nigeria, Rwanda, Sudan, Tanzania, Uganda, Zaire, and Zambia (Friis, 1992). It is native to Eritrea, Ethiopia, Kenya, Nigeria, Tanzania and Uganda. In Ethiopia, *C. macrostachyus* occurs in regions between 1300 and 2500 m with annual rainfall ranging between 750 and 2000 mm. The tree is quite persistent, regenerating large numbers of coppices or shoots, even when it is repeatedly lopped or degraded. Provided that environmental and soil conditions are favorable.

C.macrostachyus does establish well and can grow quite fast on reasonably good and well-drained soils, but prefers red or loam soils to verti soils. The latter soils are known for their shrink-swell properties (during the dry and wet seasons, respectively), and for getting waterlogged during the rainy season (Negash, 2010). *C.macrostachyusis* a large tree with cylindrical trunk. The stem is more or less pyramidal in shape with widespread branches. The stem is gray clear, smooth and fissure with age. Leaves are almost as heart-shaped large that long, they have 10 to 15 cm of length; they are flexible, green or brunette according to the season and present some prominent ribs. Flowers are regrouped in inflorescence on stems of about 25 cm of long. They are visible but their life span is very short. They are colour creamy and slightly fragrant yellow. Fruits are regrouped along an axis (Stuart and Graham, 1973*).*

Fig4 Croton macrostachyus Website http://www.prota.org date accesed22/11/08

Many parts of the *croton macrostachyus* have medicinal value including boiled leaf decoction is drunk or ashes taken orally as treatment for cough; juice from fresh leaves is applied on wounds to hasten clotting. Roots are used as an anthlemintic for tape worm, for malaria, venereal diseases, as antidiabetic, and the seeds are widely used as purgative, for constipation and for stomach worms. Bark from stems and roots is boiled in water and newly born babies are bathed in the mixture as a remedy for skin rash.The leaves of the tree are also used for fodder and the tree is used for shade. The stem bark and the tips of the different branches of the tree are used for the treatment of malaria and hepatitis indifferent parts of Ethiopia, particularly in Gojjam in the Amhara Regional State.

Chapter 3 -Materials and Methods

3.1 Study area

The studies included were in vitro studies carried out with a human bacteril pathogen. The species under the genus Croton were selected, Studies on the Croton macrostachyus Del extract constitunet and tested against common bacteria and fungi, infections by using standered drug test. The experimental study was done from April 2008 to September 2009EC.In vitro antibacterial test was conducted in Oromia public Health Research, Capacity building & quality Assurance Laboratory Center, Adama. And extraction, TLC analyses, CC, Phytochemistry Test and Extraction of volatile oil by hydro-distillation was performed in Organic Chemistry laboratory at Adama Science and Technology University, Adama. ^{1}H-NMR ^{13}C-NMR FT-IR and DEPT-135 spectra were recorded in the, NMR laboratory AAU, Ethiopia.

3.2 Plant material collection

The leaves part of the *Croton macrostachyus* was collected from Oromia Regional State, Guji Zone, Liben Woreda, particularly from Negela-Borena district, which is 600km from Addis

Ababa on April 2008. Voucher specimen is deposited in the National Herbarium, Ethiopia, Department of Biology, and AAU. (Voucher no. AD 001). It has a latitude and longitude of 50 20'N 390 35'E / 5.3330 N 39.5830 E, respectively. An altitude of about 1,475 meters above sea level and the annual average rainfall is 1800 mm with an elevation of 2,088 meters above sea level. The annual average temperature is 21°C.

3.3 Extraction and Isolation

3.3.1 Solvent extraction at room temperature

Air dried leaves powder (0.3kg) of *Croton macrostachyus* was first soaked with 1L n-hexane for 72 h at room temperature.The mixtures were filtered and the filtrate was concentrated under reduced pressure and temperature of 40^0C using rotary evaporator and afforded (3g) deep green crude extract. The marc, remaining in the extraction flask after exhaustive extraction of n-hexane, was dried and 0.3kg of powder was extracted with1L chloroform after soak-ing for 72 h at room temperature. Then the extract was concentrated in rotary evaporator and afforded (4g) deep blue crude extract. The marc remaining was then extracted using ethyle acetate, following similar procedure and yielded (5g) dark green of crude ex-tract. Lastly, 1L of methanol was added to the marc in the flask and gave (3.75g) deep black crude extract. The % yield of each extracts calculated and the result showed in (Table 1).

3.3.2 Hydro-distillation

Steam distillation is the most well-known technique for extracting essential oil from plants. In the steam distillation of essential oils, isolated using Clevenger apparatus. During the present work about 180g of powdered leaves of *C.macrostachyus* was placed into the distillation flask and steam distilled using Clevenger apparatus for 3h yielded 23.6 g (13.1%) colerless oil. The recovered mixture was allowed to settle and the oil was with-drawn (Gursale et al., 2010). After the hydro distillation process, anhydrous sodium sulfate was added to the isolated essential oil to remove residual water. The product was collected and separated using separatory funnel.

3.4 Thin layer chromatographic (TLC) analysis of crude extracts.

When each solvent extracts was subjected to thin layer chromatography (TLC) were used showed spots on TLC upon spraying 1% vanillin sulfuric acid and after heating for a few minutes were used to detect the bands on the TLC plates. The movement of the active compound was expressed by its retention factor (RF), values were calculated. On the bases of this analysis of ethyl acetate crude extract on TLC solvent system hexane: ethyl acetate (9:1) showed four colored spots. Hence, further CC would be continued when obtained the result.

TLC analysis of the ethyl acetate extract, **Insolvent system I**, hexane: ethyl acetate (10:0), 2 spots were visible Rf values 0.10 and 0.40. **In solvent system II**, hexane: ethyl acetate, (9:1), 2 spots were detected Rf values 0.82 and 0.90. **In solvent system III**, hexane: ethyl Acetate, (8:2),), 4 spots were detected RF values 0.05, 0.25, 0.80 and 0.90 **Insolvent system IV**, hexane: ethyl acetate (7:3), 2 spots were visible RF values 0.07 and 0.81. **In solvent system V,** hexane: ethyl acetate (6:4), 2 spots were obtained having RF of 0.03 and 0.94.

3.5 Isolation and purification of compounds

On the bases of TLC result a portion (5 gm) of ethyle acetate crude extract was adsorbed onto 5gm of silica gel and loaded into a chromatographic column (CC) packed with 120 g of silica gel. The column was then eluted with hexane (100%). Hexane, ethylacetate and methanol mixture gradually increasing the polarity (from 100% to 0.1%).Then, after the column was eluted using the following solvent systems; n- hexane (100%): fractions 1-5, hexane:ethyl acetate (9:1):fractions 6-13, hexane:ethyl acetate (8;2): fractions14-21, hexane:ethyl acetate (7:3): fractions *22-29,* hexane: ethyl acetate (1:1):fractions 30-31, and ethylacetate (100%): fractions32,ethylacetate:methanol(9;1)fraction*33-37*,ethyl acetate;methanol(8;2) fraction 33-37, ethyl acetate:methano(7;3), fractions 38-42, ethyl acetate:methanol(1;1), 43-47) and methanol (100%): fraction 48. Totally 48 fractions were collected each 50ml.

Out of 48 fractions which were collected using the solvent systems increased polarity? Fraction F-15 was obtained from hexane; ethyleacetate which shows single spot on TLC plate. Fractions C-34 and C-35 were combined by observing their TLC spots. In addition fraction 40 also showed characteristic coloured spots on TLC using the solvent system ethyl acetate: methanol (7:3) and upon spraying 1% vanillin sulphuric acid and after heating for a few minutes. However, the TLC examination showed twospots. Based on this TLC examination this fraction was not pure. The extract was collected by decantation and the remaining residue, which was not soluble in petroleum ether and afforded 21.25mg compound **CM-F40.** Fractionation of ethyle acetate extract on column chromatography afforded two pure compounds and only one compound is characterized as CM-F40.

3.5. Phytochemicals screening on leaves of *C.macrostachyus*

Following botanical identification of the selected plant crude extract (chloroform, ethyle acetate, methanol and essential oil) was prepared from the freshly collected material for the intended array of biological test systems. Portion of the same extract that was subjected for the biological screening was used for the identification of the major secondary metabolites, like tannins,

alkaloid, steroids, saponin and flavonoideetc were carried out according to the methods described by (Evans', 1996), Harborne, (1973, 1984), the results are presented in (Table 4)

3.5.1 Test for alkaloids: About 0.5g of the leaf extracts were stirred with about 5 ml of dilute hydrochloric acid separately and filtered. Each filtrate was tested with the following reagents:

3.5.1.1 Dragendroff's test: Few drops of Dragendroff's reagent (solution of potassiu bismuth iodide) were added to each filtrate and observed for the formation of orange yellow precipitate which may indicate the presence of alkaloids.

3.5.1.2 Mayer's test:-Few drops of Mayer'sreagent (Potassium mercuric iodide solution) were added to each filtrate and observed for the formation of white or cream colour precipitate which may indicate the presence of alkaloids

3.5.2 Test for flavonoids: To 0.5 g portion of crude extract 10 mL of ethyl acetate was added and heated with a steam bath for 3 min. The mixture was fil-tered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution and a yellow coloration was observed (Sofowora, 1993).

3.5.3 Test for Phenols: 0.5 g of each of crude extracts extracts were put in a different test tube and treated with a few drops of 2% of FeCl3; bluish green or black coloration indicated the presence of phenols (Harborne, 1998).

3.5.4 Test for Tannins: 0.5 g of each crude extract was boiled in 10 mL of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added to give brownish green or a blue-black coloration (Ayoola et al., 2008).

3.5.5 Test for Saponins :(Froth) To 0.5 g of each solvent fraction of C.macrostachyus leaves, 2 ml of chloroform was added. Then, 3ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids

3.5.6 Test for Anthraquinones: To 0.5 g of each crude extract 10 mL of benzene was added and shaken and then filtered. 0.5 mL of 10% ammonia solution was added to the filtrate and the mixture was shaken well and the presence of the violet color in the layer phase indicated the presence of the anthraquinones (Evans', 1996).

3.5.7 Test for cardiac glycosides (Keller-Killiani test) To 0.5 g of each fraction diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown

ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer

3.5.8 Test for Steroids: 0.5 g of each crude extracts was dis-solved in 5 mL of methanol. 1 mL of the extract was treated with 0.5 mL of acetic acid anhy-dride and cooled in ice. This mixed with 0.5 mL of chloroform and 1 mL of concentrated sul-phuric acid was then added carefully by means of a pipette. At the separations level of the two liquids, a reddish-brown ring was formed, as indication of the presence of steroids (Harborne, 1998)

3.5.9 Test for Terpenoids: 0.5 g of each (chloroform, methanol and water) crude powder was separately dissolved in 5 mL of methanol. 2 mL of the extract was treated with 1 mL of 2, 4-dinitrophenyl hydrazine dissolved in 100 mL of 2M HCl. A yellow-orange coloration was ob-served as an indication of Terpenoids (Sofowora, 1993).

3.6. Antimicrobial assay

3.6.1 Test Organisms:

in vitro for antimicrobial assay by using the paper disc diffusion method against. The bacterial and fungal microbes used include Staphylococcus *aureus (S. aureus)(ATTC 25923)* (gram +ve), Escheriua coli (E.coli) (ATTC 25922)(gram –ve) Klebsiella pneumoniae (*K. Pneumonia)* (ATTC700603) (gram –ve) and *Salmonella typhimurium (S. typhimurium*)(13311*) (*gram +ve) and *Candida albicans (C. albicans)* (ATCC 90028) fungus. All species were isolates obtained from patient specimens visiting Oromia public Health Research, Capacity building & quality Assurance Laboratory Center.Adama. Bacteria were maintained at 4^{o}C on nutrient agar plates before use. The fungus was maintained on inhibitory mould agar at room temperature.

3.6.2 Testing for antibacterial activity:

Antibacterial activity was used to determine the growth inhibition of bacteria by the plant extract (chloroform, ethylacetate, methanol and essential oil. The tests were carried out by using a stock concentration of 500 mg/ml prepared by dissolving 1.0 g of the extract in 2 ml of distilled water. Nutrient agar was prepared and 25 ml each was pored into sterile petri dishes. This was allowed to solidify and dry. Using a sterile cork-borer of 9 mm diameter, three holes (cups) per plate were made in the agar and they were inoculated with 0.5ml over night suspension of the bacteria/fungus. There after, the wells (holes) were filled with the extract solution. This was done in triplicate and the plates were inoculated at 27^{0}C for 18hrs. The antibacterial activities were observed and measured using a transparent ruler and recorded if the zone of inhibition was 10 mm (*Alviano et al. 2005).*

3.6.3 Testing for antifungal activity:

Sterilized paper discs were transferred to MHA plate's seeded with bacteria and incubated at 37 0C for 24 h. All the tests were performed in triplicate. The leaf crude extracts, volatile oil were taken to test the sensitivity towards four bacteria. From Whatman-No.1 filter paper discs of about 6 mm in diameter was placed in a beaker cov-ered with aluminum foil and sterilized in an oven at 180 0C for 1h. Then 10 µl and 20 µl of solution of compounds were pipetted to the discs in three replications. After allowing the sol-vent to evaporate, the paper discs impregnated with the sample solutions were then transferred with sterile forceps to potato dextrose agar seeded with spore suspension of test fungi as de-scribed above. The Petri dishes were incubated at 27 ^{0}C 3 days. The entire test was performed in duplicat *(*Lall and Meyer, 1999).

3.7 Data analysis

The results are described in the narrative and collected in the tables.

. Chapter 4 Results and Discussion

4.1 Extraction Yield The extraction yield is a measure of the solvent efficiency to extract specific components from the original material. The percentage yield of crude extract in respective solvent was recorded in Table 1. It could be calculated according to the method as follows (Zhang *et al.,* 2007).

$$\textbf{\textit{(\%)} Extracts of yield} = \frac{wt\ of crude}{wt\ of\ sample} \times 100$$

Table 1:-The percentage yield of different extracts of *C.macrostachyus* of leaves

Solvent extracts	Amount of solvent(ml)	Wt of crude extracts(g)	Extract colors	(%)yield
n-Hexane	1000	3.00	deep green	1.00%
Chloroform	1000	4.00	deep blue	1.36%
Ethyl acetate	1000	5.05	dark green	1.68%
.Methanol	1000	3.75	deep black	1.25%

Generally, different percentage yield of crude extracts were obtained from *C.macrostachyus*.The percentage yields for this plant is depicted in (Table1). In this study, the highest yield of crude extract (1.68 %) was obtained from ethyl acetate extract which followedby chioroform (1.36 %). However, the lowest yield (1.00%) was obtained from hexane (Table1). In this study.These observed variations in the presence of the phytochemicals may be due to the choice of solvent used in extraction. During extraction, solvents may have diffused into the plant material and solubilised compounds with similar polarity.

4.3 Preliminary phytochemical screening

The preliminary phytochemical screenings on leaves (chloroform, ethyleacetate and metha-nol) extracts and essential oil were tested, in this study. *Saponins, tannins, alkaloids, flavonoids, terpenoids* and *phenol.* Compounds were revealed to be present in leaves ex-tract of *C.macrootachyus* (Table 3). This shows that the plant constituents which might have high level

of medicinal values (Oloyed, 2011). This could be responsible for the versatile medicinal properties of plant.

Table 2: Preliminary phytochemical screening of solvent fractions of *Croton macrostachyus*leaves

Secondary metabolites	*Chloroform Extracts*	*Ethylacetet Extracts*	*Methanol Extracts*	*Essentialoil Extracts*
Alkaloids	+	+	+	+
Saponins	-	-	+	-
Steroids	+	+	+	+
Terpenoids	+	+	-	+
Flavonoids	-	-	+	-
tannins,	+	-	+	-
Phenols	-	+	-	-
Cardiac Glycosides	-+	+	+	-

Key :(+**)** = the presence of phytochemical constituents

(-) = the absence of phytochemical constituents

The results from the phytochemical screening revealed the presence of *tannins, saponins, alkaloids and phenols*.

The presence of pharmacologically useful substances such as *tannins, flavonoids, alkaloids, saponins* among other pharmacologically active elements (Table 2) leaf of *C.macrostachyus* as revealed by phytochemistry confirms the diverse claims and application of parts of the plant in treatment of ailments (Haristoy *et al.,* 2005). Several plants which are rich in tannins have been shown to possess antibacterial activities against a number of organism's *saponnins* though are haemolytic on red blood cells, are harmless when taken orally and they have beneficial properties of lowering cholesterol levels in the body (Amos- Tautua *et al*., 2011). *Alkaloid*s have been shown to possess both antibacterial (Erdemogli *et al*., 2009) and antidiabetic (Constantino *et al.,* 2003) properties and useful for such activities. *Phenols* and phenolic compounds have

been extensively used in disinfections and remain the standard with which other bactericides are compared (*Uwumarongie et al.,* 2007).

Thus, the antibacterial activities exhibited by the secondary metabolites: *tannins, saponins, alkaloids and phenols* may be responsible for the antimicrobial activity of the extract. However, the present result reveals that the use of *C.macrostachyus* as an antimicrobial agent is limited. Since only the methanol extracts of leaf exhibited major antimicrobial effect on the microbes used in this study as compared to the ethyleactate extracts (Tables 3) because more organic compounds were leached in this solvent. The methanol extract of leaves part of *C.macrostachyus* exhibited antibacterial activities against *Esherichia coli*. This may be due to *alkaloids* and *saponins* being largely present in the methanol leaf extract. *Alkaloids* are basically Nitrogen containing naturally occuring compounds commonly found to have antimicrobial properties due to their ability to intercalate with the DNA of microrganisms.

*Terpene*s and steroids which are mainly present in essential oils whose antimicrobial activities have been recognized for many years (Hammer *et al*., 1999). The essential oils which contain the *terpenes* and *steroids* with other compounds have been known to have diverse therapeutic uses such as the management of upper respiratory tract, urinary tract, and digestive tract infections. The *in vitro* antimicrobial screening of the extracts showed considerable inhibition against almost all the test organisms except for *E.coli* that showed resistance at ethyle acetate concentration of the extract. It can also be observed that the extracts exhibited greater zone of inhibition on S.aureus*, C.albicans* even at the lowest concentration as cmpared to the standard drug even though their concentrations are not comparable (Table 3). *Steroids* was found to be present in the all the sequential extracts and essential oil of *C.macrostachyus* leaf. However, the current study showed that *Cardiac glycoside* was present only etyle acetate and methanol crude extracts of *C.macrostachyus's* plant leaves.

The results of the present study also supplement the folkloric usage of the studied plants which possess several known and unknown bioactive compounds with bio-activity. As known in traditionally the use of this plant at local people as ''stop bleeding and healing wounds'', researchers were observed dur-ing *in vitro* phytochemical screening the *saponin* content in the plant might be high in the leaves of this plant. Because, the plant *saponins* generally help humans

to fight fungal infec-tions, combat microbes.They also bind blood cholesterol, there by reducing heart problems but the most exciting and outstanding prospect for *saponins* are how they inhibit and kill cancer cells. It has also been reported that they do so without destroying normal cells on the process, as is the mode of some cancer fighting drugs.Therefore, the phytochemical screening result reveals that the presence of these phytochemical constituents supports the use of *C.macrostachyus* medications and it is probable that these phytochemicals are responsible for the healing properties of this plant.

4.4 Structural Elucidation of Compound CM-F40

The Ethyle acetate extract of the leaves of *C.macrostachyus* was subjected to a series of chromatographic techniques, lead to the isolation of a compound CM-F40 yield (21.25mg). Com-pound CM-F40 is amorphous, deep reddish with observed melting point value of and obtained from CC of ethyle acetate extract. For isolation the best mobile phase selected by was ethyle acetate: methanol (7:3) and its RF value was determined as 0.72.

The FT-IR spectrum of the compound displayed ab-sorption bands indicating the presence of hydroxyl (3390 cm^{-1}). The ab-sorption band at 2924 cm^{-1} due to C-H stretching and absorption band at 1266 & 1044 were due to C-O stretchs

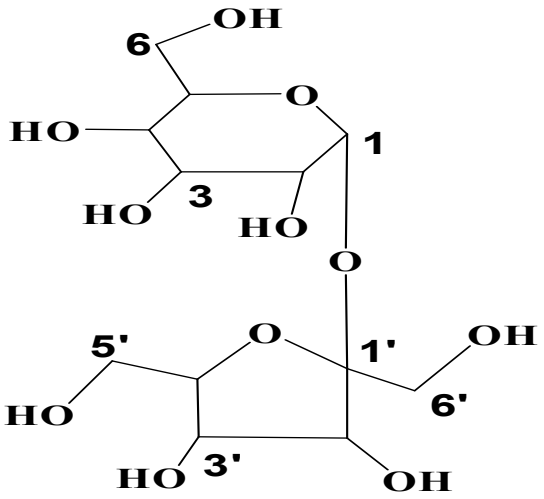
Proton NMR spectral data (Appendix 1) indicated the presence of oxygenated methine protons. The peaks at δ 5.5, δ 5.0, δ 3.4 and δ 3.1 are the peaks of oxygenated methine protons. The signals appeared as a singlet at δ 2.5 & δ 2.9 are the OH protons of the sugar.

Table3 ^{13}C NMR, DEPT-135, ^{1}H NMR spectral data of the compound CM-F40 with literature reports (Z. mobilis levansucrase 2009)

Group	Carbon No.	^{13}C-NMR d data	Reference data	DEPT-135	^{1}HNMR	Type of carbon
Glucose	1	96.87	92.81	Up	5.0	CH
	2	81.1	74.92	Up	3.1	CH2
	3	72.8	72.38	Up		CH
	4	70.6	82.02	Up		CH
	5	70.2	72.88	Up		CH2
	6	63.1	61.39	Down		CH2
Fructose	1’	62.7	61.76	down	5.5	CH2
	2’	104.5	105.80	quaternary	3.4	-
	3’	82.7	79.57	Up		CH
	4’	76.8	75.40	Up		CH
	5’	74.8	84.50	Up		CH2
	6’	63.2	64.39	Down		CH2

The ^{13}C-NMR and DEPT-135 spectra of compound CM-F40 revealed that showed the presence of 12 signals corresponding to 12 carbon atoms. Six of them were resonated at δ 96.87, 81.1, 72.8, 70.6,70.2 & 63.1 respectably were assigned to C-1, C-2, C-3, C-4, C-5,C-6, while six carbon atoms appeared at δ 104.5, 82.7, 76.8, 74.8, 63.2 & 62.7 were assigned to C- 1', C- 2', C-3, C-4', C-5', C-6' respectably. Comparing ^{13}CNMR *(*Appendix.2) and DEPT-135 *(*Appendix.3**)** spectral data.

Further confirmation of its identity was obtained from DEPT spectral data displayed three negative peaks down which indicates the presence of three CH_2 groups in the compound. All the information derived from the spectral data was characterized as tetrahydro-2-(hydroxymethyl)-6-(tetrahydro-3,4-dihydroxy-2,5-bis(hydroxymethyl)furan-2-yloxy)-2H-pyran-3,4,5-triol, whose structure was elucidated based on its ^{1}H NMR,^{13}C-NMR, DEPT-135 and by comparison with literature value (Z. mobilis levansucrase 2009).



tetrahydro-2-(hydroxymethyl)-6-(tetrahydro-3,4-dihydroxy-2,5-bis(hydroxymethyl)furan-2-yloxy)-2H-pyran-3,4,5-triol,

Figure 2 Proposed structure of Compound CM-F40.

4.5 Analysis of antimicrobial activities on *C.macrostachyus*

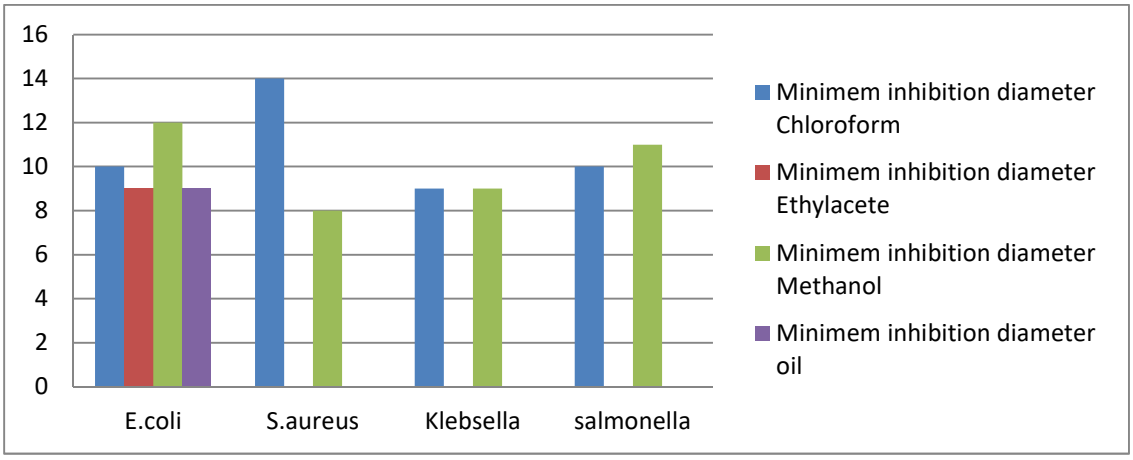
Microbial infections are great challenge to human health concern and it is even exacerbated by the growing resistance to the conventional drugs. Thus, researchers have resort to find remedy from plants for infectious diseases. Development of new antimicrobial agents is among the proposed solution to solve this problem. In this regard plants can be provided a good alternative in search for new chemicals with a wide range of antibacterial and antifungal activities (Casley-Smith, 1997). *C.macrostachyus* is traditionally known plant for healing wounds and blood clotting. Its, crude extracts and essential oil, were taken to test the sensitivity in vitro against four bacterial species (Table 4).

Table 4 Growth inhibition of C.macrostachyus extracts against selected bacterial & fungai pathogens

	E.coli	*S.aureus*	*Klebsella*	*salmonella*	*C.albicans*
Chloroform	+	+	+	+	+
Ethylacete	+	-	-	-	+
Methanol	+	+	+	+	+
Essentialoil	+	-	-	-	+

Key; (+): growth inhibition (-): no growth inhibition

Table 5 *C.macrostachyus* extracts against bacterial pathogens



The antibacterial tests showed that all of the test samples clearly indicate that a considerable antibacterial activity against the bacterial species used in the study. As whole Chloroform crude extract was found to be the most active extract for all the tested bacteria followed by methanol. The chloroform and methanol extracts were significantly active against *E. coli* and *S. aureus* had inhi-bition zones of above (12mm). The antimicrobial assay showed that volatile oil was generally less active on E. coli (8mm) than S. aureus (9mm); whereas higher antifungal activity was observed on *C.albicans* (inhibition diameter (24.20) mm).The antifungal activities of other crude extracts were followed by methnol, ethyi acetate and chloroform, respectively.

The present study clearly indicated that methanol and chloroform extract of *C.macrostachyus* could able to inhibit all test bacteria. However, the degree of their inhibition pattern is different this may be because of the difference in bacterial strain and the kind of solvent used. In this study, chloroform extract has shown the lowest inhibition zone against *Klebsella* organism and the highest inhibition zone was seen in *S. aureus*. The observed difference in antibacterial activities of extracts between Klebsella and *S.aureus* organisms may be attributed to the difference in the outer membrane of both isolates. Gram-negative bacteria possesses high permeability barrier for numerous antibiotic molecules similarly for these extracts. Their periplasmic space also contains enzymes, which are capable of breaking down foreign molecules and appears to be less susceptible to plant extracts than gram positive bacteria. (Duffy and Power, 2001)

Of all microorganisms used in this study*, S. aureus* (ST) had the highest susceptibility with chloroform extracts. Thisis an indication that the extract could be agood first line basis for drug production with high potency against infection by this bacterium. In this study most of tested bacteria showed MIC value of 15 mg/ml, followed by 30 mg/ml. This is an indication that most part of the bioactive compounds, which is found in this plant is non polar

Chapter 5 -Conclusions and Recommendation

Traditional medicine has been used and continues to be an alternative approach on treatment for various diseases. Currently, the growing interest of consumers in substances of natural origin in association with the increasing concern surrounding potentially harmful infec-tions and disease has directed to a rising interest in the use of plant extracts as functional ingredients in many pharmaceutical products.

As described in literature review based on the physiological activity of the plant secondary metabolites classified as saponins, tannins, terpenes, alkaloids, flavonoids, etc. The phytochemical screening tests for the major secondary metabolites were performed in this research work. From the study, the leaves extract was found to contain saponins, tannins, terpenoids, phenols, alkaloids and steroids and cardiac glycoside. The presence of these important phytochemicals in the plant is a scientific justification of the plant use in the traditional treatment against various diseases affecting humans and animals. The presence of these phytochemicals in this plant enhances their pharmaceutical and therapeutic potentials.

.

The antimicrobial properties of *Croton macrostachyus* based on relevant research reports.Solvent extracts of *C.macrostachyus* inhibited both Gram positive and Gram negative bacteria. But ethyle acetate and essential oil inhibited the growth of the antibiotic sensitive strain of E.coli. Essential oil obtained by hydro-distillation inhibited some strains of the studied bacteria and fungai enhanced the effect of some of the antibiotics used in the studies.

The antimicrobial activities of this extracts could be attributed to the presence of bioactive agents, which are either non-polar or semi polar, including, among others, tannins, alkaloids, saponins, flavonoids, steroids, and terpenoids that act individually or collectively. The results show that the extracts of *C.macrostachyus* could be used as alternative means in pharmacotherapy of bacterial infections. They could be also used as adjuvant agents in antibiotic therapy against pathogenic bacterial infections (Nascimento *et al*., 2000).After successive solvent extraction and column chromatography two compounds were isolated from the plant leaves. From these isolated compounds, only one compound coded as CM-F40 characterized as tetrahydro-2-(hydroxymethyl)-6-(tetrahydro-3,4-dihydroxy-2,5bis (hydroxymethyl) furan-2-yloxy)-2H-pyran-3,4,5-triol,

As the researchers' observed during analysis of chemical component, the plant is rich with different secondary metabolites.. Further studies are needed on this plant un-der study to characterize and elucidate structures of more bioactive compounds. In addition to this the following recommendations were made.

- There is need for conducting more studies to identify and characterize the medicinal principles in the tested this plant, which may serve as novel compounds for development of new and more effective antimicrobial drugs. This would prove very useful especially in this era when drug resistance is a major issue
- This plant should be studied more extensively to explore its activity on other or-ganisms like other bacteria species, protozoa and helminthes among others.
- Toxicity studies of the plant should also be done to determine the safety indices of the extract
- InVivo studies of the fractions should be done to possible mechanism of actions

.

Reference

1. Thomas, G. **2000**.Medicinal Chemistry, An Introduction, John Wiley and sons, Ltd., Chichester, England,(pp(.1).
2. Williams, D. A.; Lemke, T. L., **2002**. Foye's Principles of Medicinal Chemistry, Fifth edition, Lippincott Williams and Wilkins, Philadelphia, pp.(24).
3. TODD, A., **1960**.Natural Product Chemistry-Retrospect and Prospect, Chemistry of NaturalProducts International Symposium, Australia, pp.(359).
4. Koskinen, A,.1993.Asymmetric Synthesis of Natural Products, John Wiley and Sons Ltd. Chichester, England,(pp.(1). 169-171.
5. Salatino. A., Salatino MLF. Negri.G., **2007**.. Traditional uses, chemistry and pharmacology of *Croton* species (Euphorbiaceae). *J Braz Chem Soc,pp,(18*0 11-33.
6. Nguefack J,. **2004.** Five essential oils from aromatic plants of Cameroon: their antibacterial activity and ability to permeabilize the cytoplasmic membrane of Listeria innocua examined by flow cytometry. Lett. Appl.Microbiol.(pp39),395-400.
7. Kouki, A., Haataja. S., **2011**. Identification of a novel streptococcal adhesin P (SadP) protein recognizing galactosyl-α1-4-galactose-containing glycoconjugates: convergent evolution of bacterial pathogens to binding of the same host receptor. J Biol Chem.; pp286 (45):38854-64.
8. McChesney JD, 1991. Antimicrobial diterpenes of Croton sonderianus. II. ent-Beyer-15-en-18-oic acid. Pharm Res.(pp 8(10):1243-7.
9. Vlietink, A.J., van Hoof, L., Totte, J., 1995. "Screening of Hundred Rwandes Medicinal Plants for Antimicrobial and Antiviral Properties". J. Ethnopharm.pp(46).31-47.
10. Nascimento GGF., **2000**. Antibacterial activity of plant extracts and phytochemicals on antibiotic resistant bacteria. Braz. J. Microbiol pp(31).247-56.
11. Abo KA, Ogunleye VO, Ashidi JS., **1999**.Antimicrobial potential of Spondias mombin, Croton zambesicus and Zygotritonia crocea. Phytother Res.pp13(6):494-7.
12. Cowan, M.M. 1999. Clinical microbiology review. American Society for Microbiology, 2(4): 567-569.
13. Ahmad, I., Aqil, F. and Owais, M. **2006**. Mordern phytomedicine turning plants into drugs. Willey-VCH verlag GMbH and Co KGaA, Weinhei.
14. Just, M.J., Recio, M.C.,**1998.** Antinflammatory activity of unusual lurpine saponins from Bupleurum fruticescens. Thieme-E Journals, 64: 404-407.

15. Leandro, L.M. and Vargas, F.S**. 2012**.Chemistry and Biological activities of terpenoids from Copaiba (Coparfera SPP). Oleoresins, 17: 3866-3889.
16. Wendakoon C, Sakaguchi M. Inhibition of amino acid decarboxylase activity of Enterobacter aerogenes by active components in species. J Food Prot. 58; 280-283.
17. Stern, J.L., **1996**. Phlorotannin protein inte-ractions. Journal of Chemical Ecology, 22: 1887-1899.
18. Negash L. **2010**.A Selection of Ethiopia's Indigenous Trees: Biology, Uses and Propagation Techniques. Addis Ababa University Press, Addis Ababa, Ethiopia pp 386.
19. Karunamoorthi K. and Ilango K. (2010): Larvicidal activity of Cymbopogon citratus (DC) and Croton macrostachyus against Anopheles arabiensis Patton, a potent malaria vector. Eur Rev Med Pharmacol Sci; 14:57-62.
20. Rodrigues FF., **2009**.Synergy effects of the antibiotics gentamicin and the essential oil of Croton zehntneri. Phytomedicine. 16(11):1052-5.
21. Alviano DS, Bizzo HR, Souto-Padrón T, Rodrigues ML, Bolognese AM, Alviano CS, Souza MM**., 2005.** Antimicrobial activity of Croton cajucara Benth linalool-rich essential oil on artificial biofilms and planktonic microorganisms. Oral Microbiol Immunol.;20(2):101-5.,
22. Peres MT, Monache DF, Cruz AB, Pizzolatti MG, Yunes RA. ,**1997.** Chemical composition and antimicrobial activity of Croton urucurana Baillon (Euphorbiaceae). J Ethnopharmacol. 56(3):223-6.
23. Liu WJH. Introduction to Traditional Herbal Medicines and their Study, in Traditional Herbal Medicine Research Methods: Identification, Analysis, Bioassay, and Pharmaceutical and Clinical Studies. John Wiley & Sons 2011.
24. Chariandy CM,.1999.Screening of medicinal plants from Trinidad and Tobago for antimicrobial and insecticidal properties. J Ethnopharmacol.; 64(3):265-70
25. Bum E.N., Ngah E.**2012**. Epilepsy Behav. 24: 319.
26. Friis I. (1992): Forests and forest trees of northeast tropical Africa. Their natural habitats and distribution patterns in Ethiopia, Djibouti and Somalia. Kew Bulletin, Additional Series, XV. Her Majesty's Stationery Office, London pp.396
27. Gilani, A.H. and Rahman, B. 2005. Perspective paper: Trends in ethnopharmacology. Journal of Ethnopharmacology, 100: 43-49.
28. Kaufman, P. B.; Cseke, L. J.; Warber, S.; Duke, J. A.; Brielmann, H. L., 1999. NaturalProducts from plants, CRC Press, Washington, D. C, pp. 1, 9-11.
29. Kumar Choudhury M. h., Shiferaw Y. and Hussen. A (2013): Toxicity of Millettia ferruginea darasana (family: Fabaceae) against the larvae and adult ticks of Amblyomma variegatum Fabricius a three-host tick in cattle. J Parasit Dis DOI 10.1007/s12639-013-0311-8.
30. Gursale, A., Dighe, V. and Parekh, G. 2010. Simultaneous Quantitative Determination of Cin namaldehyde and Methyl Eugenol from Stem Bark of Cinnamomum zeylanicum Blume Using RP-HPLC. Journal of Chromatographic Science, 48: 59-62

31. Sofowora, A. 1993. Medicinal Plants and Traditional Medicine in Africa. Spectrum Books Ltd., Ibadan. p. 289.
32. Taniguchi M, Kubo I. **1993.** Ethnobotanical drug discovery based on medicine men's trials in the African savanna: screening of east African plants for antimicrobial activity II. J Nat Prod. 56(9):1539-46.
33. Santos, A.O., Ueda-Nakumura, T., Dias Fiho, B.P., Veiga, V.F., Pinto, A.C. and Nakamwa. C.V., **2008**. Antimicribila activity of Brazilian Copaipa oils obtained from different species of the Copalifera genes. Mem Inst. Oswaldo Cruz, 103: 277-281
34. Sarker, S.D., Latif, Z. and Gray, A.I. 2006. '*Natural Product Isolation'*, in *Natural Product Isolation* 2nded, Human Press, Totowa, New Jersey. pp. 1-25
35. Shalid M, Tayyab M, Naz F, Jamil A, Ashraf M, Gilani AH., **2008** Activity-guided isolation of a novel protein from *Croton tiglium* with antifungal and antibacterial activities. Phytother Res. 22(12):1646-9
36. Argal, A. and Pathak, A.K. 2006. CNS activity of Calotropis gigantean roots. Journal of Eth nopharmacology,pp(19) 425-428.
37. Ayoola, G.A., Coker, H.A., 2008.Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. Tropical Journal of Pharmaceutical Research, 7(3): 1019-1024.
38. Lall N, Meyer JJ 1999. In vitro inhibition of drug-resistant and drug-sensitive strains of Mycobacterium tuberculosis by ethnobotanically selected South African plants. J Ethnopharmacol. 66(3):347-54.
39. Zhang, S., Bi, H. and Liu, C. 2007. Extraction of bio-active components from Rhodiola sacha linensis under ultrahigh hydrostatic pressure. Separation and Purification Technology, 57: 275.
40. Eka, O.U. 1998. Roots and Tuber Crops in Nutritional Quality of Plant Foods. Osagie A. Eka O.A.(Eds) Post harvest Res. Unit Publication, University of Benin. pp. 1-31.
41. Mendoza, L., Wilkens, M. and Urzua, A. 1997. Anti-microbial study of resinous exudates and of diterpenoids and flavonoids isolated from some Chilean pseudognaphalium (Asteraceae). Journal of Ethnopharmacology, 58: 85-88.
42. Casley-Smith J. R. 1997. Coumarin in the treatment of lymphoedema and other high-protein oedemas. In R. O'Kennedy and R. D. Thornes (Ed.) Coumarins: biology, applications and mode of action (p. 348). New York: John Wiley and Sons, Inc.
43. Evans' W.C. 1996. Trease and Evans Pharmacognosy, 14th edition, WB Sauders company Ltd; London. pp. 224–228, 29 –309, 542–575.
44. Trease GE, Evans WC., **1989.** Textbook of Pharmacognosy. 12th Edn, Balliere, Tinadl London, *Akerele, O., **1984**. Whose traditional medicine program: progress and perspectives. WHO Chron; pp 38(2):76-81.*
45. *Duru S, Grierson DS, Afolayan AJ., **2006.** Antimicrobial activity of Solanum aculeastrum. Pharm Biolpp; 44:283–286.*

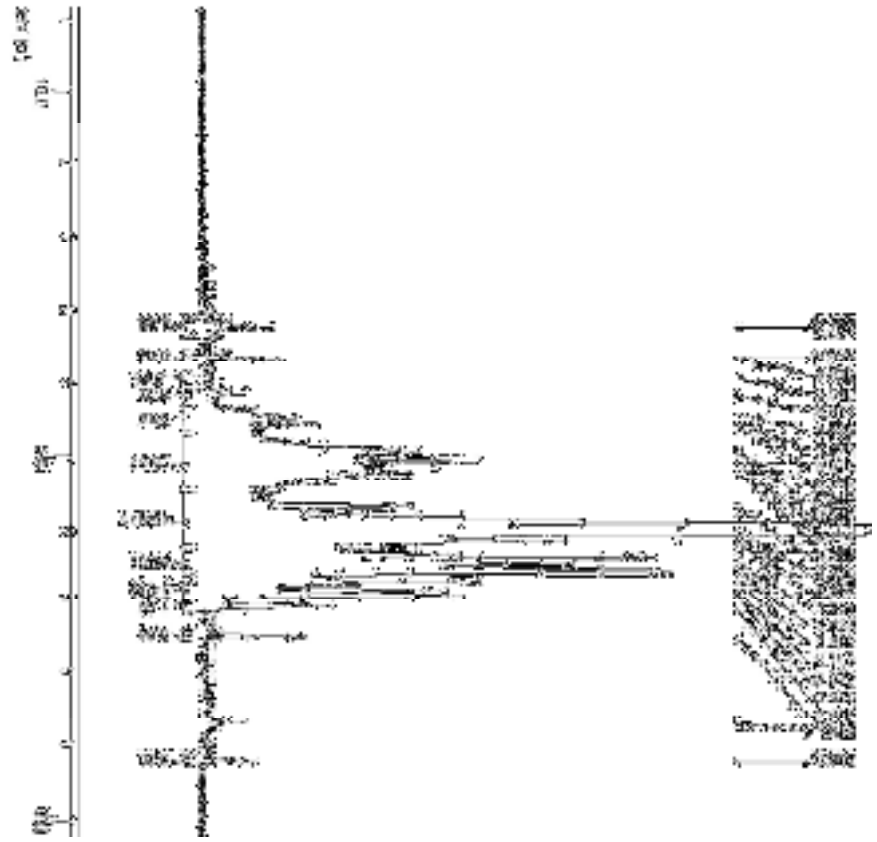
46. *Williams, D. A.; Lemke, T. L.* ***2002.*** *Foye's Principles of Medicinal Chemistry, Fifth edition,*
Lippincott Williams and Wilkins, Philadelphia, pp. 244.
47. *Abebe, D., Ayehu, A.,* ***1993.*** *Medicinal plants and enigmatichealth practices of Northern Ethiopia, Berhanina Selam Printing Enterprise, Addis Ababa, Ethiopia, pp 341.*
48. *Murthy, P.N., Moges, G., Hymete A, Gebremariam T.,* ***1993.*** *Antimicrobial and phytochemical screening of Justicia Schimperiana. Eth Pharm pp 11:47-53.6..*
49. *Getahun A., 1976. Some common medicinal and poisonousplants used in Ethiopian folk medicine. Addis AbabaUniversity, Addis Ababa,pp 3-5.*
50. *Abebe D.,* ***1996.*** *The role of Herbal Remedies and theApproaches To wards their Development, In Proceedings of the Workshop on Development and Utilization of Herbal Remedies in Ethiopia, Nazareth, Ethiopia, pp 111:29.*
51. *Teferi G.,* ***2003****. The use of medicinal plants in self-care inrural Ethiopia. J Ethnopharmacol 87:155-161.*
52. *Kokate, CK.* ***2000****. Practical Pharmacognosy. Edn 4, VallabhPrakashan, New Delhi, India, pp 107-111.* Schmidt E, Jirovetz L, Buchbauer G
53. Denkova Z, Stoyanova, A, Murgov I., **2005**. Antimicrobial testing and gas chromatographic analysis of aroma chemicals.J. Essential Oil Bearing Plants; 8:99-106
54. Vila, J., M. Vargas, H. Urassa, H. Mshinda, D. Schellemberge, and J. Gascon. 1999. Antimicrobial resistance of diarrhoeagenic *E. coli* isolated from children under age 5 years from Ifakara, Tanzania. Antimicrobial Agents and Chemotherapy, 43(12): 3002-3004
55. *Harbone J.B.,.* ***1998****.Phytochemical Methods: A Guide toModern Techniques of Plant Analysis. Edn 3, Chapman & Hall, London, UK, pp 60-66.*
56. *Egwaikhide, P.A., and Gimba, C.E.* ***2007.****Analysis of the phytochemical content and anti- mi-cro bial activity of Plectranthus glandulosis whole plant. Middle-East Journal of Scientific Research, pp 2(****3-4):*** *135-138.*
57. *Derwich, E., Benziane, Z., Chabir, R. and Taouil, R.* ***2011****. In Vitro Antibacterial Activity and Analysis of the Essential Oil Extract of Leaves of Rosmarinus officinalis grown in Mo rocco. International Journal of Pharmacy and Pharmacological Science, pp 3(****3****): 89-95.*
58. *Nwinyi, O., Chinedu, N.O.O., Ajani, O., Ikpo Chinwe and Ogunniran, K.O.* ***2009.*** *Antibac terial effects of extracts of Ocimumgratissimum and piper guineense on E. coli and S. aureus. African Journal of Food Science, pp 3(3): 077-081.*
59. *Duffy, C, and Power, R.* ***2001****. Antioxidant and antimicrobial properties of some Chinese plant extracts. International Journal of Antimicrobial Agents.pp* ***17(6****): 527-529.*
60. *Kaufman, P. B.; Cseke, L. J.; Warber, S.; Duke, J. A.; Brielmann, H. L., 1999. Natural Products from plants, CRC Press, Washington, D. C., pp. 1, 9-11.*

61. *Thomas, G., 2000. Medicinal Chemistry, An Introduction, John Wiley and sons, Ltd., Chichester, England, pp1.*
62. *Koskinen, A.; **1993**.Asymmetric Synthesis of Natural Products, John Wiley and Sons Ltd. Chichester, England, pp. 9**1**,) 169-171.*
63. *Mann, J.; Davidson, R.S.; Hobbs, J. B.; Banthrope, D. V.; Harborne, J. B. **1994**. Natural Products: their chemistry and Biological Significance, Addison Wesley LongmanLtd, England, pp. **2**, 289, 293.*
64. *Torssell, K. **1997.** B. G. Natural Product Chemistry, A mechanistic, biosynthetic and ecological approach, second edition, Apotekarsocieteten, Stockholm, Sweden, pp. 16-17.*
65. Nakanishi, K.; Goto, T.; Ito, S.; Natori, S..,**1974.**,Nozoe, S. Natural Produts Chemistry, *Volume 1, Kodansha Ltd, Tokyo, pp. 2-4.*
66. *Rohmer, M.; Seemann, M.; Horbach, S.; Bringer-Meyer, S.; Sahm, H., J **1996.**ournal of American chemical Society, Vol. **118**.2564.*
67. *Bernfeld, P,. **1963**. Biogenesis of Natural Compounds, the Macmillan Company, New York, , pp**. 641.** 644, 657- 658 37.*
68. *Dewick, P.M., **2004.**Medicinal Natural Products, A Biosynthetic Approach, Second Edition, John Wiley and Sons, Ltd. Chichester, England, pp. 167-168.*
69. *Thomson, R. H. **1993.**The Chemistry of Natural Products, Second Edition, Blackie Academic and professional, an imprint of Chapman and Hall, UK, pp. (**106)**. 107, 111.*
70. *Duvold, T., Pale-Grosdemange, C.; Rohmer, M., **1997.**Tetrahedron Letters, Vol. (**38)**No. 28, 4769.*
71. Chung, K.T., Wong, Y.T., Wei, C.I., Huang, Y.W. and Lin, Y. 1998. Tannins and human health. *Critical Reviews in Food Science and Nutrition*, 38(6): 421-464
72. *Francis, F. J. **2000.**Wiley Encyclopedia of Food Science and Technology, Second Edition, Volume 1, John Wiley and Sons, Inc. New York, pp.(**275).***
73. *Das K., Tiwari RKS,. Shrivastava DK. **2010**. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. Journal of Medicinal Plants Researchpp4 **(2)** 104-111.*
74. Alper. J.,1998. Effort to combat microbial resistance lags. ASM news,pp(64). 440-441.
75. Yibralign Z. Master‟s thesis on Phytochemical Investigation on the stem bark of Croton macrostachyus (Bisana). Addis Ababa University 2007.
76. Balunas, M.J. and Kinghorn, A.D. 2005. Drug discovery from medicinal plants. *Journal of Life Sciences*, pp(78). 431-441.
77. http:/www. York. ac. uk/res/celp/webpages/projects/ecology.
78. Stratton, C.W., and R.C. Cooksey, 1991. Susceptibility tests: special tests, p. 1153-1165.
79. Borris, R. P. 1996. Natural products research: perspectives from a major pharmaceutical company. Journal of Ethnopharmacology, 51:29-38.
80. Burt S. **2004.**Essential oils: their antibacterial properties and potential applications in foods– A review. *Int. J. Food Microbiol*,; 94:223-53**.**

81. WHO (**2002**). Traditional medicine growing needs and potential. WHO Policy Perspectives on Medicines, World Health Organization, Geneva, pp. 1-6.
82. Matias EF, Santos KK, Almeida TS, Costa JG, Coutinho HD., **2011**. Phytochemical prospection and modulation of aminoglycoside antibiotic activity by *Croton campestris* A. Chemotherapy. 57(4):305-9.
83. Okemo, P.O., S.M. Kariuki and P.W. Ndung‟u. 2004. Multi-drug resistance in *E.coli*, *Salmonella* and *Shigella* causing diarrhoea in children below 5 years in Kenyan hospitals. The Second Regional Microbiology Conference. Journal of Tropical Microbiology and Biotechnology, Nairobi, Kenya. 2: 28-35.
84. van Ee BW, Riina R, Berry P., **2011.** A revised infrageneric classification and molecular phylogeny of New World *Croton* (Euphorbiaceae). *Taxon 60*: 1-33.
85. Fauci, A. **1998.** New and reemerging diseases: The importance of biomedical research. Emerging Infectious Diseases. (www.cdc.gov/ncidod/EID/vol4no3/fauci). 4:3.
86. Casley-Smith J. R. 1997. Coumarin in the treatment of lymphoedema and other high-protein oedemas. *In* R. O'Kennedy and R. D. Thornes (Ed.) Coumarins: biology, applications and mode of action (p. 348). New York: John Wiley and Sons, Inc.

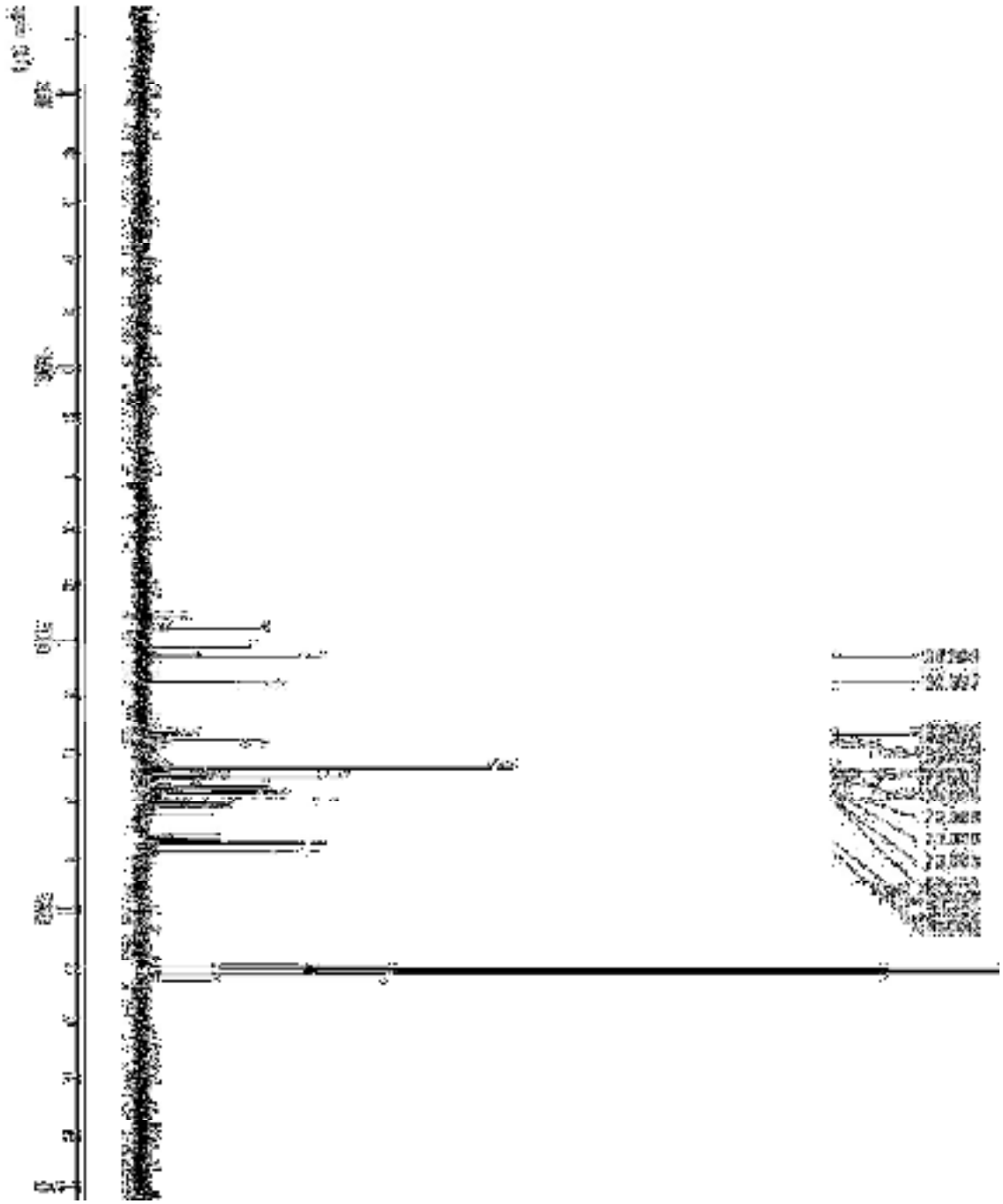
APPENDIX

APPENDIX 1:- 1H-NMR spectrum of compound 1



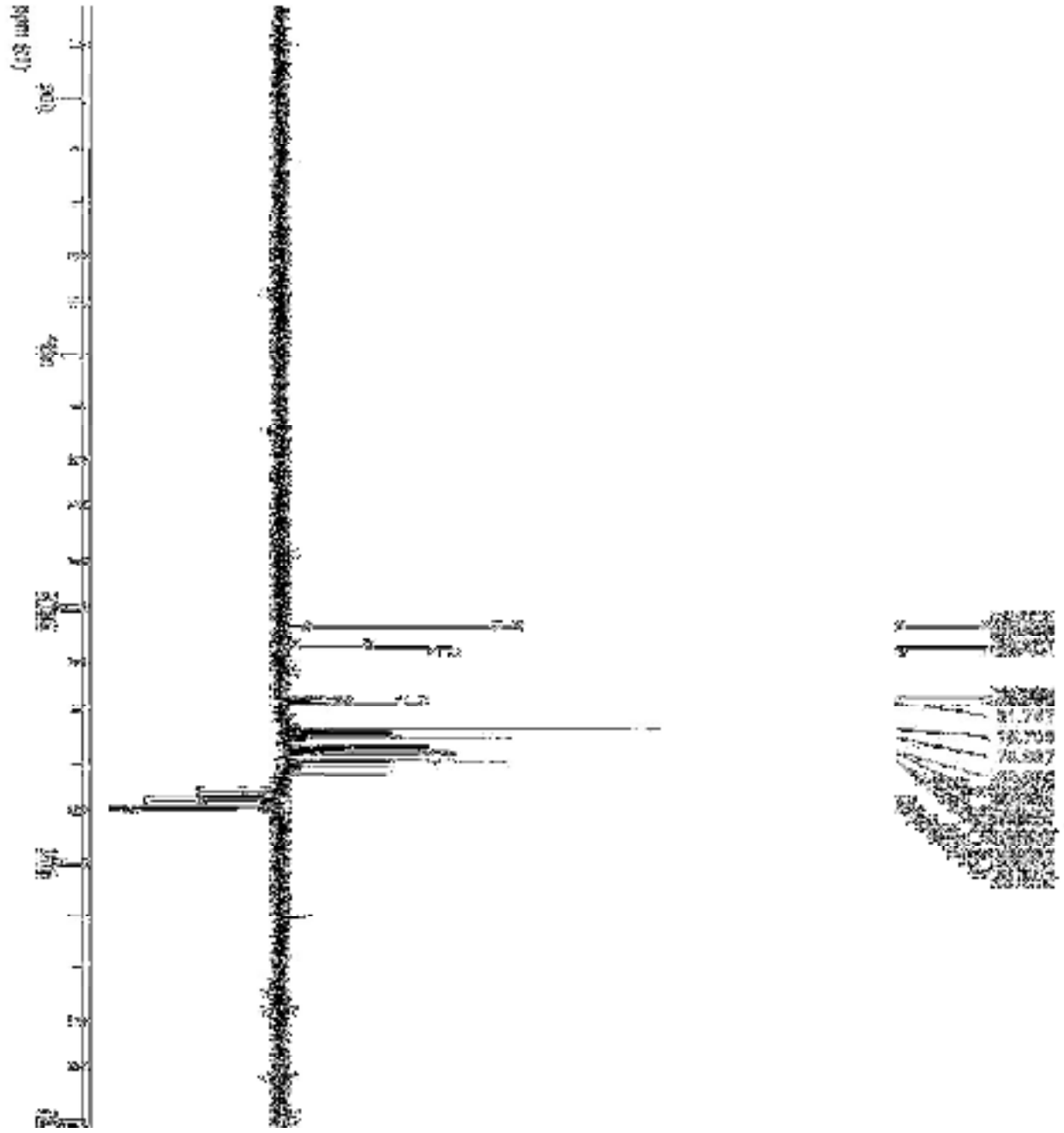
APPENDIX 2:

13C-NMR spectrum of compound 1



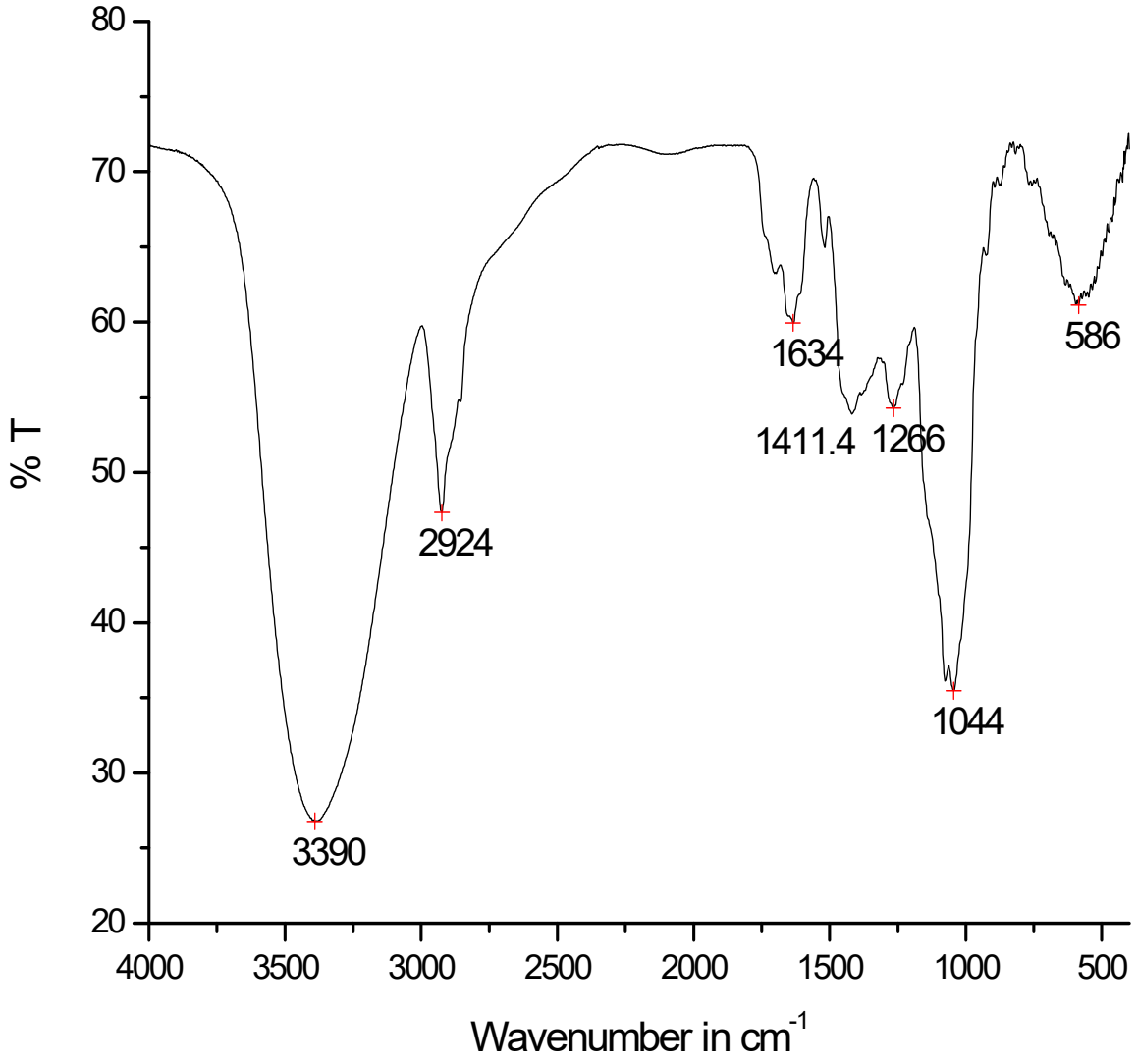
APPENDIX 3:

DEPT-135 spectrum of compound 1



APPENDIX 4:

IR spectrum of F-40



APPENDIX 4: IR spectrum of compound CM-F14

: Plant Collection form

Name of plant	**Local name of plant**	**Parts of plant collected**	**place of collection**
Croton macrostachyus Del	**bissana**	**Leaf**	**Negalle borena**

2: Pictures of plant used in the chemistry and biology Activity tests

3: Pant material preparation

 Collected plant part were washed with distilled water to remove dirt and soil particles.

 The plant were cut into small pieces, spread out on paper sheets, dried in shaded area at room temperature. Completely desiccated plant part powdered finely.

4: Crude extraction procedure

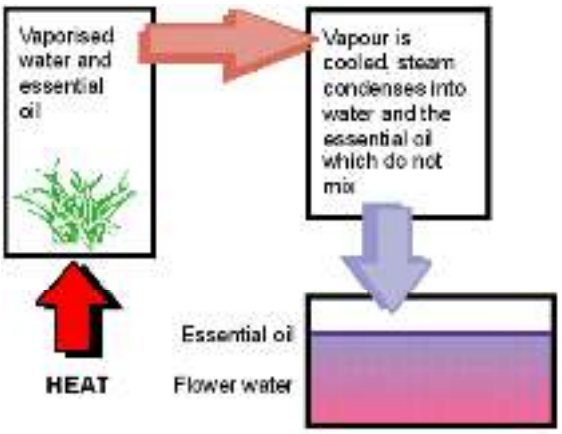
✓ Powdered plant material Weighed, transferred in separate flask and extracting agent added .

✓ Each flask contain plant material and extracting agent shaken for 24h by automatic orbital shaker.

✓ The mixture was later strained using a muslin cloth and filtered using a Whatman filter paper (No. 1: 125mm) .

✓ The filtrate was concentrated in a vacuum rotary evaporator and was evaporated to dryness in an air oven at 40°C .

5: Oil extraction method


Vaporised water and essential oil
Vapour is cooled, steam condenses into water and the essential oil which do not mix
Essential oil
Flower water
HEAT